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Short communication

Gaseous allyl isothiocyanate to inhibit the production of aflatoxins, beauvericin and enniatins by *Aspergillus parasiticus* and *Fusarium poae* in wheat flour

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ABSTRACT

There is a growing concern about the presence of mycotoxins in foods, since up to 25% of cereals and cereal foods are contaminated with these compounds. Moreover, the general public is against the use of synthetic preservatives in foods and the use of natural antimicrobials in foods is a current trend. Allyl isothiocyanate (AITC) is a volatile antimicrobial derived from oriental and black mustard. The objective of this work was to evaluate the capacity of gaseous AITC in inhibiting the production of mycotoxins by *Aspergillus parasiticus* (aflatoxin producer) and *Fusarium poae* (beauvercin and enniatin producer) in wheat flour. Petri dish lids filled with 2 g of wheat flour were inoculated with 10^4 conidia/g of *A. parasiticus* or *F. poae* and placed in a 1 L mason jar. AITC was added at 0.1, 1 or 10 μL/L in the gaseous phase. Jars were hermetically closed and kept at 23 °C for 30 d. Mycotoxins were identified and quantified by LC-MS/MS. Even 0.1 μL/L of AITC was able to produce 6.9–23% reduction of mycotoxin production. In general, synthesis of aflatoxins and beauvericin was more affected than enniatins. The use of AITC at 10 μL/L completely inhibited the production of all mycotoxins for 30 d. AITC at low doses could be added to flour packages in order to inhibit the production of potentially dangerous mycotoxins.

1. Introduction

There is a growing concern about mycotoxin contamination in food and animal feed, as these metabolites can be found in a wide range of commodities, including cereals, cereal products, spices, dried fruits, wine and coffee. The Food and Agricultural Organization (FAO) has estimated that approximately 25% of cereals are contaminated with high levels of mycotoxins (Duarte, Lino, & Pena, 2010), which is a major challenge faced by producers. The contamination of these products by fungi and, consequently, by mycotoxins is influenced by environmental factors such as temperature, humidity, and the extent of rainfall during cultivation, harvesting and post-harvesting (Campbell, Molyneux, & Schatzki, 2003).

Aflatoxins are some of the most prevalent mycotoxins in cereals. These compounds are characterized as polyketide secondary metabolites produced by some species of the *Aspergillus* genus, particularly *Aspergillus parasiticus* and *Aspergillus flavus* (Cheraghali et al., 2007). Aflatoxin B_1 is the most potent natural carcinogen known and is usually the major aflatoxin produced by toxigenic strains. The liver is the primary target to this toxin and exposure to AFB_1 in the diet is considered an important risk factor for the development of primary hepatocellular carcinoma (Kensler, Roebuck, Wogan, & Groopman, 2011). The International Agency for Research on Cancer has classified AFB_1 as a Group 1 human carcinogen (IARC, 1993).

Many countries have established maximum tolerable levels on mycotoxins occurrence in foods due to the increasing awareness of their harmful carcinogenic, mutagenic and teratogenic effects in humans and animals alike (Set & Erkmen, 2010). In 1998, the Commission of the European Community set the maximum level for AFB_1 at 2–5 ng/g for various food products (Moss, 2002), where wheat flour can contain a maximum of 2 ng/g of AFB_1. In Brazil, the maximum tolerable level of AFB_1 in food products range from 1 to 20 ng/g, and it presents the same maximum tolerable level

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for wheat flour as the European Union. On the other hand, the US allows a maximum of 20 ng/g of total aflatoxins in most kind of foods, including wheat flour.

Fusarium species are commonly found on cereals grown in the temperate regions of America, Europe and Asia (SCF, 2002). Species of the *Fusarium* genus such as *Fusarium avenacum*, *Fusarium proliferatum*, *Fusarium poae*, *Fusarium tricinctum*, *Fusarium subglutinans* are the main producers of the emerging mycotoxins enniatins A, A_1, B, B_1 (ENA, ENA_1, ENB and ENB_1, respectively) and beauvericin (BEA) in various cereals, especially wheat, barley and maize (Jestoi, 2008; Nazari, Sulyok, Kobarfard, Yazdanpanah, & Krska, 2015). Enniatins (ENs) and BEA possess a cyclic hexadepsipeptide structure and present similar toxic activity, including the induction of apoptosis, increase cytoplasmic calcium concentration and lead to DNA fragmentation in mammalian cell lines (Lin et al., 2005).

In recent years, there is a growing interest in the use of natural antimicrobials for pathogen control due to the usually accepted safe status of these compounds (Luciano & Holley, 2009). Isothiocyanates (ITCs) are reported as some of the most potent antimicrobials from plant origin. These compounds are formed after the hydrolysis of glucosinolates, which occur naturally in cruciferous vegetables, such as broccoli, cabbage, cauliflower, kale, turnip, radish, canola, rapeseed and various mustard species (Zhang, 2004). Allyl isothiocyanate (AITC) has been applied as a fumigant agent to avoid mold growth and mycotoxin production in pizza crust (Quiles, Manyes, Luciano, Mañes, & Meca, 2015a), nuts (Hontanaya, Meca, Luciano, Mañes, & Font, 2015) and wheat tortillas (Quiles, Manyes, Luciano, Mañes, & Meca, 2015b). The isothiocyanate group (–N=C=S) has a strongly electrophilic carbon that enables ITCs to readily bind to thiol and amino groups of amino acids, peptides and proteins forming conjugates (Cejpek, Valusek, & Velisek, 2000). Therefore, this compound is able to inhibit essential microbial enzymes such as thioredoxin reductase, acetate kinase (Luciano & Holley, 2009) and cytochrome *c* oxidase (Kojima & Ogawa, 1971). Moreover, AITC was shown to cause damage in the cell membrane of microbes, leading to leakage of cellular metabolites (Lin, Preston, & Wei, 2000).

The objective of this study was to evaluate the use of gaseous AITC to inhibit mycotoxin production by *A. parasiticus* (AFs producer) and *F. poae* (BEA and ENs producer) inoculated in wheat flour.

2. Materials and methods

2.1. Chemicals

BEA, ENs (A, A_1, B, B_1) and AFs (B_1, B_2, G_1, G_2) (98% purity), phosphate buffer saline (PBS) at pH 7, formic acid (HCOOH) ammonium formate (NH_4HCO_2) and AITC (>95% purity) were obtained from Sigma–Aldrich (St. Louis, USA). Acetonitrile and methanol were purchased from Fisher Scientific (New Hempshire, USA). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath.

2.2. Fungi strains and culture conditions

F. poae ITEM 9175 was purchased from the Istituto delle Scienze delle Produzioni Alimentari del Consiglio Nazionale delle Richerche (ISPA-CNR, Bari, Italy) and *A. parasiticus* CECT 2681 were obtained from the Spanish Type Culture Collection and by the (CECT Valencia, Spain). The fungi grew in Petri dishes containing potato dextrose agar (PDA) for 7 days at 25 °C. Suspensions containing of *F. poae* ITEM 9175 or *A. parasiticus* CECT were prepared by harvesting isolated colonies from potato dextrose agar (PDA) and adding the fungal material in 5 mL of potato dextrose broth (PDB). Conidial concentration was measured by optical density at 600 nm and adjusted to 10^6 conidia/mL (Kelly, Grimm, Bendig, Hempel, & Krull, 2006).

2.3. AITC antifungal activity and mycotoxin reduction in wheat flour

Petri-dish lids (50 mm diameter) containing 2 g of white wheat flour were incoculated with either *F. poae* ITEM 9175 or *A. parasiticus* CECT 2681 at ~10^4 conidia/g. Lids were placed in 1 L glass jars (Analisis Vinicos, Spain) (Fig. 1). Filter papers of 2.5 × 2.5 cm containing 0.1, 1 or 10 μL of liquid AITC (>95% purity) were freshly prepared and inserted into the jars, which were hermetically closed. AITC promptly volatilizes at room temperature, which acts as a fumigant. The control group did not receive any treatment. Jars were kept at room temperature (23 °C) for 30 d. Then, the flour was autoclaved and used for mycotoxin analysis.

2.4. AFs, BEA and ENs extraction from wheat flour

Each wheat flour sample was transferred to a 50 mL centrifuge tube and combined with 0.1 g of sodium chloride (NaCl) and 20 mL of a methanol/water mixture (60:40 V/V). Samples were extracted using an Ika T18 basic Ultraturrax (Staufen, Germany) for 5 min. The extracts were centrifuged at 3500 rpm during 5 min at 4 °C and a 10 mL aliquot of the supernatant was completely dried using a nitrogen evaporator Turbovap LV (Zymark, Runcorn, UK) operating at 30 °C and 5 psi nitrogen pressure. The dried extract was resuspended in 1 mL of methanol and filtered through a 0.22-μm filter (Phenomenex, Torrance, CA, USA) before toxin identification and quantification by liquid chromatography (LC) coupled with a mass spectrometry detector (MS) (Liu et al., 2013).

2.5. LC-MS/MS analysis of AFs, BEA and ENS

The liquid-chromatography analysis system consisted of a binary LC-20AD pump, a SIL-20AC homoeothermic auto sampler, a CTO-20A column oven and a CMB-20A controller and an Analyst Software 1.5.2 was used for data acquisition and processing. The separation of AFs was performed on a Zorbax SB-C18 column (150 × 5 mm, I.D, 3.5 μm; Agilent, CA, USA) at room temperature (20 °C). The mobile phase was composed of solvents A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) at a flow rate of 0.4 mL/min. After a hold time of 0.6 min, 10% of B reached 95% in 1.6 min and was kept constant for 0.3 min. Afterward, the column was re-equilibrated with 10% solvent A until the end of the run at 4.0 min. Separation of ENs and BEA occurred in a Gemini column (150 × 2 mm, 5 μm, Phenomenex) and the mobile phase was composed by acetonitrile (70%) and water with 0.1% formic acid, which was run isocratically at 0.2 mL/min. An API-4000 triple-quadruple MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with ESI interface in positive mode was used for detection of all mycotoxins in multiple reactions monitoring (MRM) mode. The main MS parameters were optimized and finally set as follows: nebulizer gas (GS1), 55 psi; auxiliary gas (GS2), 750 psi; curtain gas (CUR) 15 psi; capillary temperature 550 °C; ion spray voltage (IS) 5500 V. Nitrogen was used as the nebulizer, heater, curtain and collision gas. The precursor-to-product ion transitions were *m/z* 313.3/285.1, *m/z* 315.3/259.0, *m/z* 328.7/243.3, *m/z* 330.9/257.1 and *m/z* 386.2/122.2 for AFB1, AFB2, AFG1, AFG2, respectively. For the detection of BEA the precursor ion was *m/z* 801 and the product ions selected were m/z 784 and 756. For ENB the precursor ion was *m/z* 640, and the product ions were *m/z* 196 and 527. For ENB1 the precursor ion was m/z 654,

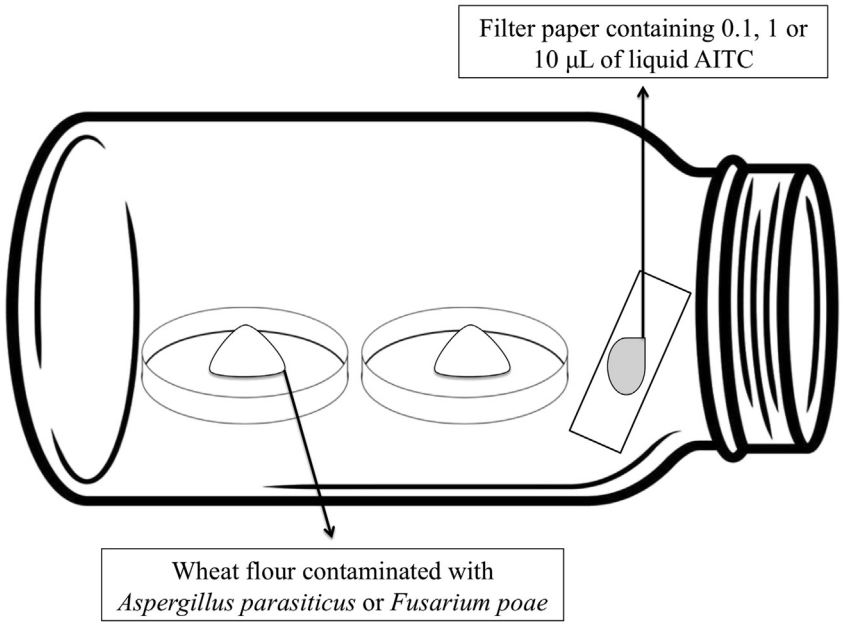

Fig. 1. Scheme of the fumigation process. The wheat flour contaminated with either *Aspergillus parasiticus* or *Fusarium poae* was added to petri dish lids and AITC was added to an adjacent filter paper. The jar was hermetically closed and volatilization of the oil occurred at room temperature.

and the product ions *m*/*z* were 196 and 228, for ENA the precursor ions was *m*/*z* 682, and the product ions were *m*/*z* 210 and 555, for ENA1 the precursor ion was *m*/*z* 668, and the product ions were *m*/*z* 210 and 541.

2.6. Validation procedures

Validation of the analytical method was performed according to established by the EC (EC657/2002). Recovery and standard deviations (RSDs) of mycotoxins were measured by adding AFs, ENs and beauvericin in wheat flour at levels of 50 and 250 ng/mL, in five different days (inter-day) and five replicates (intra-day), previously evaluated for endogenous mycotoxins. The precision of the method was determined by repeatability and reproducibility studies, and expressed as RSD (%). The limits of detection (LOD) and quantification (LOQ) were calculated based on signal: noise ratio of 3:1 and 10:1, respectively. Linearity was evaluated by calibration curves (triplicate) using six concentrations, ranging from lowest calibration level (LCL) to 100 times LCL. Matrix effects were investigated by comparing the slopes of standards diluted in solvent with the slopes of matrix extract matched standards.

2.7. Statistical analysis

All experiments were performed three times in duplicates ($n = 6$) for each fungal strain and AITC doses. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnet's multiple comparison tests. Differences were considered significant if $p < 0.05$.

3. Results and discussion

3.1. AFs, BEA and ENs reduction in wheat flour

The method validation data for mycotoxins determination in wheat flour are summarized in Table 1. Analytical method showed good performance, with recoveries varied from 71 to 82%. Repeatability ranged from 7 to 20% for intra and inter-day experiments, always lower than 20% for both spiked levels (50 and 250 μg/kg). LOD and LOQ ranged from 0.03 to 7.00 μg/kg and 0.1–20.0 μg/kg respectively. For legislated mycotoxins (AFs), the LOQs were lower than the Maximum Levels (MLs) established by the European Union (EC 401/2006). Matrix effect was higher to enniatins (A, A_1, B, B_1) and while LCL was similar among the mycotoxins evaluated ($R^2 > 0.983$).

Quantification of AFs, BEA and ENs in the wheat flour contaminated with *A. parasiticus* CECT 2681 and *F. poae* ITEM 9175 and treated with AITC is presented in Fig. 2. All mycotoxins were produced at high levels (mg/Kg) and AITC was able to inhibit their production in a dose-dependent manner. BEA was found at the highest concentration (55.5 mg/kg) among all mycotoxins analyzed in control samples, whereas the lowest concentration was found for AFB1 at 24.2 ± 1.0 mg/kg. Overall, *A. parasiticus* produced 125.8 ± 7.7 mg/kg of AFs and *F. poae* produced 183.0 ± 8.4 mg/kg of ENs plus BEA in the control samples. AITC was able to inhibit the production of AFs by 19.9, 45.29 and 100% when applied at 0.1, 1 and 10 μL/L, respectively. Similarly, production of BEA and ENs was inhibited by 15.4, 47.7 and 100% with 0.1, 1 and 10 μL/L of AITC, respectively. The capacity of AITC in inhibiting the production of AFs by *A. parasiticus* was also observed in pizza crust, where 10 μL/L of AITC was also able to avoid totally the synthesis of AFs (Quiles, Manyes, Luciano, Mañes, & Meca, 2015). The lowest dose of AITC used by these authors was 2 μL/L, which reduced AFs production roughly by 50%. Therefore, it seems that AITC fumigation could be suitable to inhibit the production of harmful mycotoxins in different food matrices.

As presented on Table 2, concentrations as low as 0.1 μL/L of AITC were able to inhibit mycotoxin production in up to 23%. The least affected mycotoxin at this dose was ENB_1, which was reduced by only 6.9%. Overall, synthesis of ENs by *F. poae* was less affected than AFs production by *A. parasiticus* at 0.1 μL/L of gaseous AITC. Application of 1 μL/L AITC resulted in a more homogenous inhibition of mycotoxin synthesis that ranged between 35.9 ± 2.0 and 55.1 ± 3.4, whereas 10 μL/L AITC totally inhibited the production of all mycotoxins. Isothiocyanates are extremely reactive molecules that can inhibit fungal growth by inhibiting essential metabolic pathways

Table 1
Performance characteristics of LC-MS/MS method.

Mycotoxin	Spiked level (μg/Kg)	Recovery[a] (%)	Intra-day (% RSD)	Inter-day (% RSD)	LOD (μg/Kg)	LOQ (μg/Kg)	LCL[b] (ng/mL)	R^2	ME[c] (%)
Aflatoxin B_1	50	75	7	12	0.08	0.2	1.2	0.983	73
	250		9	10					
Aflatoxin B_2	50	74	8	12	0.08	0.2	1.2	0.999	69
	250		9	11					
Aflatoxin G_1	50	71	12	14	0.16	0.5	1.2	0.990	70
	250		10	13					
Aflatoxin G_2	50	72	13	15	0.30	0.9	1.2	0.994	77
	250		9	11					
Beauvericin	50	73	11	16	7.00	20.0	1.5	0.999	70
	250		14	20					
Enniatin A	50	82	8	9	2.50	7.5	1.0	0.997	79
	250		10	10					
Enniatin A_1	50	80	7	10	0.50	1.5	1.0	0.996	89
	250		7	8					
Enniatin B	50	80	8	10	0.03	0.1	1.0	0.998	90
	250		11	10					
Enniatin B_1	50	79	10	17	0.06	0.2	1.0	0.997	82
	250		9	19					

RSD: relative standard deviation, LOD: limit of determination, LOQ: limit of quantification, LCL: lowest calibration level, R^2: correlation coefficient, ME: matrix effect.
[a] Mean of recovery of 5 replicates each level evaluated at 5 different days, $n = 20$.
[b] Minimum level of calibration curve ranged until ME 100×.
[c] Matrix effect evaluated as follow: (slope matrix matched calibration/slope standard in solvent) x 100.

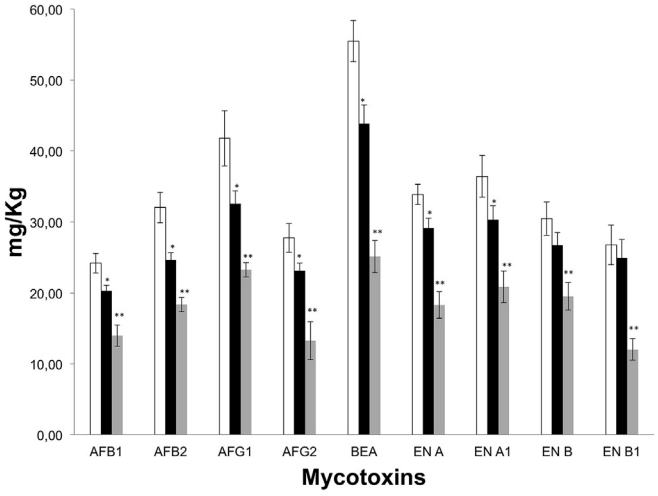

Fig. 2. Mycotoxin production by *Aspergillus parasiticus* (AFs) and *Fusarium poae* (BEA and ENs) in wheat flour with the absence (white) or presence of AITC (black = 0.1 μL/L, gray = 1 μL/L). No mycotoxin was detected at 10 μL/L of AITC (not shown). Data are expressed as mean ± SD (n = 3). Significant differences in comparison to the control were expressed as $^{*}P < 0.05$ and $^{**}P < 0.01$.

Table 2
Reduction of AFs production by *Aspergillus parasiticus*, and BEA and ENs production by *Fusarium poae* growing in wheat flour and treated with 0.1, 1 and 10 μL/L of AITC.

Mycotoxins	% of reduction			
	Control	0.1	1.0	10.0
AFB_1	0.0	16.2 ± 1.3	42.3 ± 2.0	100.0
AFB_2	0.0	23.0 ± 2.2	42.7 ± 1.9	100.0
AFG_1	0.0	22.1 ± 3.2	44.4 ± 3.1	100.0
AFG_2	0.0	16.5 ± 2.5	52.3 ± 2.9	100.0
BEA	0.0	21.0 ± 2.6	54.7 ± 3.5	100.0
EN A	0.0	14.1 ± 3.1	46.1 ± 2.6	100.0
EN A_1	0.0	16.8 ± 1.3	42.7 ± 1.8	100.0
EN B	0.0	12.2 ± 1.0	35.9 ± 2.0	100.0
EN B_1	0.0	6.9 ± 0.6	55.1 ± 3.4	100.0

(Kojima & Ogawa, 1971; Luciano & Holley, 2009) and disrupting the microbial plasmic membrane, leading to the leakage of cytoplasmic metabolites (Ahn, Kim, & Shin, 2001). Moreover, AITC was also shown to react with mycotoxins such as BEA (Meca, Luciano, Zhou, Tsao, & Mañes, 2012) and fumonisins (Azaiez, Meca, Manyes, & Fernández-Franzón, 2013), but the toxicity of the reaction products *in vivo* has yet to be determined. Nonetheless, this antimicrobial agent seems to be a great candidate to avoid mycotoxins in foods by both inhibiting their synthesis and changing their chemical structure once they were already produced.

Although gaseous AITC at 0.1 and 1 μL/L were able to inhibit the production of AFs by *A. parasiticus*, the final concentration of total AFs found in the wheat flour were well above the limit allowed by the Brazilian (5 μg/kg), European (4 μg/kg) and US (20 μg/kg) legislations. The only practical concentration of AITC that would keep AFs levels within these legislations was 10 μL/L. BEA and ENs are emergent minor *Fusarium* mycotoxins that have yet to be legislated, but their cytotoxicity has already been described (Lin et al., 2005). As for AFs, total inhibition of BEA and ENs synthesis by gaseous AITC was only reached at 10 μL/L. Since AITC is a very pungent compound, it can alter the sensory characteristics of foodstuffs. The minimum recognizable concentration of AITC used as a gaseous preservative in rye bread and hot dog bread were 2.4 mg/L and 1.8–3.5 mg/L, respectively (Nielsen & Rios, 2000). These concentrations are 3–5 times lower than the necessary to totally inhibit AFs, ENs and BEA synthesis in wheat flour. However, wheat flour is not generally consumed raw. Normally, it is used as an ingredient of several food products that undergo a thermal process. AITC is very volatile and might be present in the final food product at extremely low doses, which may not be detected by the human palate.

4. Conclusion

Gaseous AITC at 10 μL/L was able to completely inhibit the production of AFs, BEA and ENs by *A. parasiticus* and *F. poae* inoculated in wheat flour. This is a natural compound found in mustard, wasabi and horseradish that could be used to preserve foods against fungal spoilage and to increase food safety by inhibiting mycotoxin synthesis. Further studies should analyze the impact of

adding AITC in wheat flour packaging on the flavor characteristics of foods prepared with the treated flour.

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